Transmission Rates of Transgenic *Asaia* Bacteria through Multiple Generations of *Anopheles gambiae s.s.* (Diptera: Culicidae)

Research Thesis

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by

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Abstract

Vector-borne diseases impact millions each year and as a result many endemic countries face slow economic growth and enormous health costs to treat these diseases. It has been well documented that resistance to insecticides and drugs is spreading. Recently, the use of genetically modified vectors has been one proposed method of control, and while generating a refractory vector has been accomplished, a method for replacing the wild population has not been found. Another possible pathway for controlling the disease is to genetically alter the bacterial gut symbionts found in the vectors, thus providing an effector molecule to inhibit the disease in the wild population. In this study, we conducted two separate experiments to determine if the genetically modified bacteria could be transmitted to the offspring 1) in an environment that was free of antibiotics and 2) where naturally occurring microbes would be present. A portion of an adult *Anopheles gambiae* population was infected with *Asaia* bacteria that had been genetically modified to express a red fluorescent protein (DsRed). The mixed population was then released, the females allowed to blood feed and oviposit, and the offspring reared to adulthood. The results from the experiments indicated that naturally occurring microbes could be interfering with the transmission of the modified *Asaia* or that vertical transmission was insufficient to provide enough bacteria to successfully inoculate the offspring.

Introduction

Malaria is arguably the most important vector-borne disease worldwide, affecting 300 million people and killing one million people annually, primarily young children (Breman et al., 2001; Bonnefoy et al., 2008). Vector-borne diseases have traditionally been fought with drugs and insecticides such as chloroquine and DDT, but we are now struggling to stay ahead of resistance in both malaria and in mosquitoes. One approach has been the development of transgenic refractory vectors, which is a method of inserting the genes required to provide a natural resistance in mosquitoes to malaria. While new refractory vectors are being generated, the mechanism to replace wild vector populations remains elusive (Riehle and Jacobs-Lorena, 2005). The largest hurdle to the use of refractory vectors, aside from a mechanism for replacing the susceptible population, is the presence of multiple reproductively isolated strains of *Anopheles gambiae* Giles (Diptera: Culicidae), which requires the development of multiple refractory strains (della Torre et al., 2002). One new approach is to use acetic acid bacteria, which are naturally found in the midguts of various insect vectors, to produce an effector molecule that will inhibit the vector-borne disease.

Acetic acid bacteria, such as *Asaia*, *Acetobacter*, and *Gluconacetobacter*, form a symbiotic relationship with several orders of arthropods including, Hymenoptera, Hemiptera, and Diptera (Crotti et al., 2010). There are two different forms of symbiotic relationships; the obligate primary symbionts and the facultative secondary symbionts (Crotti et al., 2009). These bacteria can affect the reproduction, development, and fitness of the host (Dale and Moran, 2006; Feldhaar and Gross, 2009). The symbionts are transmitted or acquired through a variety of pathways including feeding on nectar or transmission from father to mother then the offspring (Crotti et al., 2010). The transmission from mother to offspring is through a direct method of egg-smearing or by an indirect method of contaminating the environment from which the larvae

are then infected (Favia et al., 2007; Crotti et al., 2010). The multiple transmission routes make the use of facultative symbionts an ideal candidate for genetic manipulation to increase a natural resistance to parasites or to insert into the vector new characteristics for resistance to parasites and viruses.

Genetic manipulation of bacterial symbionts is favored over development of transgenic refractory vectors for both technical and ethical reasons (Damiani et al., 2010). The genetic manipulation of bacteria is cheaper and faster than introducing genes into mosquitoes. Bacteria are also easier to transport and can be introduced into the environment on a greater scale with a faster effect on the reduction in the size of the malaria reservoir in the mosquito population. The use of bacterial symbionts could be implemented on a shorter timescale due to regulations on the release of genetically modified bacteria already existing (Sayler and Ripp, 2000). These benefits make the use of bacterial symbionts a better choice in the fight against malaria and offer a novel technique in controlling the spread of malaria and other vector-borne diseases that affect human health and agriculture.

The acetic acid α-proteobacterium belonging to the genus *Asaia* has been isolated from the midgut, salivary glands and reproductive organs of the Malaria vector *Anopheles stephensi* Liston (Favia et al., 2007). The locations where *Asaia* are found in the mosquito provide two major benefits for affecting malaria in the vector population. Malaria is taken up by the mosquito as male and female gamete forms, and once inside the gut of the mosquito, an oocyst forms. From this oocyst, the sporozoite form of Malaria emerges and travels to the salivary glands. The presence of *Asaia* in close proximity to the malaria plasmodium within the gut provides an opportunity to introduce an effector molecule that could inhibit the plasmodium from being able to either attach to the epithelial cells of the gut or by killing the parasite. The term effector

molecule, here, will be used to indicate a molecule that is capable of interfering with the transmission or lifecycle of a vector-borne disease. The location of *Asaia* in the reproductive organs indicates that *Asaia* could be transmitted both vertically and horizontally to other members of the population (Damiani et al., 2008). In *An. stephensi*, genetically modified *Asaia* are transmitted from males to females during mating and *Asaia* DNA was found on the eggs, larvae, and pupae (Favia et al., 2007).

In the present study, which was designed to determine whether the methods of Favia et al. (2007) are practical and effective, *An. gambiae* were inoculated with genetically modified *Asaia* (DsRed) and placed in an antibiotic free environment where naturally occurring *Asaia* and other bacteria would also be present. The ability of *Asaia* to colonize the reproductive organs and subsequently be transmitted to the offspring was examined.

Materials and Methods

Insect:

The study mosquito, *Anopheles gambiae* sensu strict, Mbita strain, was obtained from a colony established by the staff of the International Centre of Insect Physiology and Ecology (ICIPE) in 2001 from a population of *An. gambiae* in Mbita Point, Kenya. The colony has been maintained in the vector behavior laboratory of The Ohio State University (70% ± 5% RH, 26ºC ± 2ºC, and 12:12 (L:D)) since 2006. Colony adults were provided with water and a 10% sucrose solution continuously, and were given a human blood meal once per week. One day after each blood-feeding, a cup of tap water for oviposition was placed inside the cage.

<u>Experiment 1:</u> Determining Asaia transmission rates in simulated natural conditions

Insect:

Experimental mosquitoes were reared beginning with 100 recently hatched larvae per pan (23 x 33 x 5 cm) with 450 ml of aged tap water for a total of 6 pans. The larvae were fed powdered Tetramin™ fish food according to a daily regimen described by Gary and Foster (2001). Pupae were placed in a small mouse cage (20 x 26.5 x 14.5 cm) until emergence and the adults were provided with water. Approximately 220 adult males and 220 adult females were separated into additional small laboratory cages and were provided water and a 2% sucrose solution. Forty-two *An. gambiae* adult females and males were then provided with a 12.5% sucrose solution containing 1 optical density (OD) of *Asaia* (DsRed) until it was observed that the mosquitoes had ingested the solution by the indication of an expanded crop. The twenty-two male and female inoculated mosquitoes were then released into a small mesocosm (see below) along with 110 males and 110 females that had not been provided with the *Asaia* solution. The remaining uninoculated 100 adult males and 100 adult females were placed in the vector behavior laboratory as a control for the experiment.

Bacterium:

The study bacterium *Asaia* was obtained from cultures maintained by David Lampe at Duquesne University. The DsRed-tagged bacteria were constructed by insertion of the DsRed gene into the chromosome of *Asaia sp.* and used in the bacterial colonization experiments as a stable recombinant that does not need antibiotic selection (Damiani et al. 2008; Mølbak et al. 2007). All

recombinant bacteria were grown for 24 h at 30°C in GLY medium (25 g l−1 glycerol, 10 g l−1 yeast extract, pH 5)

Environment and Testing Procedure:

The experiment was performed in a small mesocosm (11.1 m^3). Three species of plants were placed into the mesocosm, each species bearing extra-floral nectarines that provided sugar for the mosquitoes. The species were *Ricinus communis* L., *Tecoma stans* L., and *Senna occidentalis*. The mesocosm also contained two pots as resting sites placed in opposite corners on the floor and eight artificial sugar-feeding sites: four suspended, two on the ground, and two inside the resting sites. The feeding sites contained a 10% honey solution. A human blood meal (WAF) was offered on the day of inoculation and again the following day. One large oviposition site, a pan (47.6 x 33.7 x 7 cm) containing aged tap water was placed in the middle of the mesocosm. The ambient temperature was 25-37°C, with an average of 29°C. The room humidity was 48-85% with an average of 75%. The resting site temperature range was recorded as 26-34°C with an average of 29°C. The humidity range recorded in the resting site was 62-80% with an average of 69%. Once eggs were oviposited, they were collected and the larvae reared according to the methods previously mentioned. The larvae were fed powdered Tetramin fish food according to a daily regimen described by Gary and Foster (2001). A sample of 20 males and 20 females were collected from the mesocosm along with 20 females and 18 males from the control population and shipped to David Lampe at Duquesne University in Pittsburg, PA, to test for the presence of *Asaia* and the DsRed gene. Additionally, 25 adult male and female F_1 progeny, 61 4^{th} instar larvae, and 50 pupae also tested for the presence of *Asaia* and the DsRed gene.

Experiment 2: ***Determining* Asaia *transmission rates in cage studies***

Insect:

Two hundred mosquitoes were raised to adults according to the previously mentioned rearing methods. Fifty adult males and 50 adult females were separated into a small laboratory cage and were provided water and a 2% sucrose solution. All 100 *An. gambiae* adult females and males were then provided a 12.5% sucrose solution containing 1 OD of Asaia (DsRed) until it was observed that the mosquitoes had ingested the solution by the indication of an expanded crop. The inoculated mosquitoes were then transferred into a small cage (30 x 30 x 46 cm). Additionally, 110 males and 110 females were placed into the above mentioned incubator, in separate small laboratory cages, for use as a control.

Environment and Testing Procedure:

Experiment 2-Part A: Transmission rates of Asaia to F_1 progeny from 100% P_1 colonization

The experiment was performed in a small cage placed inside a temperature controlled incubator. Inside the cage were one resting site, one feeding site containing aged tap water, and one sugar-feeding site containing a solution of 10% sucrose. The first 24 hours after the mosquitoes were released into the cage, a solution containing 12.5% sucrose and 1 OD of *Asaia* (DsRed) was provided instead of a 10% sucrose solution. A human blood meal (JDG) was offered the day of inoculation and again the following day. One oviposition cup containing aged tap water was placed in the cage. The temperature inside the cage was 23.2-26.0°C with an average of 25.3°C and the humidity range was 50.7-77.1% with an average humidity of 66.2%. Again, eggs were reared to adults at 100 larvae per pan. A sample of 20 inoculated males and 20 inoculated females, eggs, 50 1st instar larvae, and a water sample were collected, along with 20 females and

20 males from the control population, and 20 adult male and female F_1 progeny. The samples were then shipped to David Lampe to test for the presence of *Asaia* and the DsRed gene.

Experiment 2-Part B: Determining transmission through consumption of egg remnants

A group of 10 females was removed 24 hours after the samples from experiment 2 part A was sent for testing and placed into a small mouse cage located in the incubator. A human blood meal (JDG) was offered to both groups of female mosquitoes and an oviposition cup containing aged tap water was placed in both cages. The oviposition cup from the small mouse cage contained 0.1 mg of Tetramin fish food and distilled water, whereas the oviposition cup from the cage contained only distilled water. A sample of 20 1st instar larvae from the oviposition cup containing food and 20 1st instar larvae along with 20 pupae from the oviposition cup containing only distilled water were tested for the presence of *Asaia* and the DsRed gene.

Results

***Experiment 1:* Determining Asaia transmission rates in simulated natural conditions**

Differences in the colonization of *Asaia* between the parent generation and the F_1 generation were observed. The results from the first inoculation indicate that the DsRed-tagged *Asaia* are able to colonize *An. gambiae* at a rate as high as 85% after just one feeding (Table 1). The first experiment also indicated that the colony of mosquitoes from which the specimens were obtained was also infected with a wild-type (wt) strain of *Asaia*. The total number of individuals colonized with *Asaia* (wt) in our control group was 28 out of 48 (58.3%), while the total number of individuals colonized with *Asaia* (DsRed) in our test group was 33 out of 40 (82.5%).

Sex	Inoculated	Generation	Life Stage	Colonized	DsRed
Female	No	P_1	Adult	10/20 (50%)	0/20 (0%)
Male	No	P_1	Adult	18/28 (64%)	0/28 (0%)
Female	Yes	P_1	Adult	20/20 (100%)	16/20 (80%)
Male	Yes	P_1	Adult	19/20 (95%)	17/20 (85%)

Table 1: Summary of the colonization experiment 1 of *An. gambiae* with DsRed-tagged *Asaia* sp. after inoculation.

The colonization of the F_1 progeny showed that *Asaia* (wt) did colonize a small portion of the adults (Table 2) with the total number being 9 out of 50 (18%). Differences were seen in the colonization between the adults and the pupae and larvae. The pupae and larvae indicated no colonization by *Asaia*. This difference in rate indicates that *Asaia* was present in the environment but that it was not obtained until the mosquitoes became adults. The F_1 generation of adults, pupae, and larvae were not colonized by the DsRed-tagged *Asaia*.

Sex	Generation	Life Stage	Colonized	DsRed
Female	F_1	Adult	5/25 (20%)	0/25 (0%)
Male	F_1	Adult	4/25 (16%)	0/25 (0%)
	F_1	Pupal	0/50 (100%)	0/50 (0%)
	F_1	Larval 4th Instar	0/67 (95%)	0/67 (0%)

Table 2: Summary of the colonization experiment 1 of *An. gambiae* with DsRed-tagged *Asaia* sp. for F_1 generation.

***Experiment 2:* Determining Asaia transmission rates in cage studies**

Experiment 2-Part A: Transmission rates of Asaia to F_1 progeny from 100% P_1 colonization

Asaia (wt) was again found to have colonized the control group (Table 3) with a total number of colonized individuals being 28 out of 39 (71.7%). This was in contrast to the test group which indicated a colonization of 32 out of 40 (80%) individuals. The number of individuals with the DsRed-tagged *Asaia* was lower, with 24 out of 40 (60%) carrying the DsRed gene. The eggs and

the oviposition water sample were positive for colonization and for the DsRed gene. The 1st instar larvae showed no colonization of *Asaia*.

Sex	Inoculated	Generation	Life Stage/Sample	Colonized	DsRed
Female	No	P_1	Adult	11/20 (55%)	0/20 (0%)
Male	No	P_1	Adult	17/19 (89%)	0/19 (0%)
Female	Yes	P_1	Adult	12/20 (60%)	8/20 (40%)
Male	Yes	P_1	Adult	20/20 (100%)	16/20 (80%)
		F_1	Larval 1st Instar	0/50 (0%)	0/50 (0%)
		F_1	Eggs	Yes	Yes
			Oviposition Water	Yes	Yes

Table 3: Summary of the colonization experiments 2 part A of *An. gambiae* with DsRed-tagged *Asaia* sp. after inoculation of parent generation and F_1 generation larvae and eggs.

Experiment 2-Part B: Determining transmission through consumption of egg remnants

The F_1 progeny showed a colonization rate of 100%, but none of the individuals were colonized by the DsRed-tagged *Asaia* (Table 4). The second cohort of F_1 progeny showed colonization of 11 of the 20 larvae provided food in the oviposition cup, and among the larvae from the oviposition cup with only distilled water the number of individuals showing colonization was 10 out of the 20 tested. The pupae from the oviposition cup with food showed no colonization. In all three groups, there was no growth from the DsRed-tagged *Asaia*.

Sex	Food	Inoculated	Generation	Life Stage	Colonized	DsRed
Female		No	F_1 Cohort 1	Adult	20/20 (100%)	0/20 (0%)
Male		No	F_1 Cohort 1	Adult	20/20 (100%)	0/20 (0%)
	Yes		F_1 Cohort 2	Larval 4th Instar	11/20 (55%)	0/20 (0%)
	Yes		F_1 Cohort 2	Pupal	0/20 (0%)	0/20 (0%)

	No		F_1 Cohort 2	Larval 4th Instar	10/20 (0%)	0/20 (0%)

Table 4: Summary of the colonization experiment 2 part B of *An. gambiae* with DsRed-tagged *Asaia* sp. for F_1 generation cohort 1 adults and F_1 generation cohort 2 larvae and pupae.

Discussion

Previous studies looking at using *Asaia* to infect several generations through vertical transmission, as reviewed in the introduction, have been conducted in an artificial environment making it difficult to come to a firm conclusion about the effectiveness of relying on vertical and horizontal transmission as a means to spread genetically modified gut symbionts in *An. gambiae* (Damiani et al., 2010; Favia et al. 2007). This study provides some evidence to support the ability of *Asaia* to colonize a population of adult *An. gambiae* through a single feeding, but this study also provides evidence that the vertical transmission of *Asaia* is not completely effective.

When determining the ability of *Asaia* to colonize the mosquitoes through a single feeding, an issue arose in that the colony used to obtain specimens was already colonized by a wild-type *Asaia.* DNA-based analysis indicated that the DsRed-tagged *Asaia* was able to infect the adult mosquitoes after just one feeding with a rate of up to 85%. The presence of the wild-type *Asaia* did contribute to our simulated natural conditions and any pressure from the wild-type on the introduced *Asaia* may have contributed to the inability for the DsRed-tagged *Asaia* to be passed on to the next generation via vertical transmission. The use of aged tap water in the experiments to simulate natural conditions may also have contained additional bacteria and other microbes that may have also inhibited transmission of the DsRed-tagged *Asaia*. In experiment 1, only 22 of the 264 mosquitoes were inoculated with the DsRed-tagged *Asaia.* The low percentage of inoculated individuals also may have been a reason for the lack of transmission, and in experiment 2 we sought to determine if this could have been a factor.

In part A of experiment 2 all 100 individuals were inoculated with the DsRed-tagged *Asaia*. The rate of colonization of the DsRed-tagged *Asaia* was as high as 80% in the inoculated adults. Using this percentage, it was shown that both the eggs and the water from the oviposition cup did contain DsRed-tagged *Asaia*. Though the desired *Asaia* was in the egg and water sample, the presence of *Asaia* was not indicated in the sample of 1st instar larvae.

The lack of *Asaia* in the sample of the 1st instar larvae indicated that they did not ingest the bacterium, and thus their guts were not colonized by, *Asaia*. The mechanism for the ingestion of the *Asaia* as described by Damiani et al. (2010) is the consumption of the egg after the larvae hatch. The *Asaia* has been hypothesized as being smeared onto the surface of the egg as it exits the reproductive tract (Damiani et al. 2010; Favia et al. 2007). In experiment 2 part B, the ingestion of the eggs, and thus *Asaia,* was tested. A group of females from the parent generation that had been inoculated with DsRed-tagged *Asaia* was separated, and the oviposition cups in the two groups contained either distilled water or distilled water and 0.1 mg of Tetramin fish food. This experiment indicated that having no food other than the egg chorion did not increase the rate of transmission. In both groups, the presence of *Asaia* was confirmed but neither group tested positive for the DsRed-tagged *Asaia*. This would indicate that either the larvae were not ingesting their egg chorion or that the amount of *Asaia* smeared onto the egg was not sufficient to colonize the larvae.

The experiments showed that vertical transmission of *Asaia,* or at least the strain used here, may not be an efficient method for introducing a genetically modified *Asaia* strain that would produce an effector molecule for inhibition of malaria. Additional experiments should be conducted to determine the feasibility of the egg-smeared *Asaia* to colonize the larvae and to determine if the larvae could lose the symbiont while molting.

Environmental acquisition seems to be the most effective method in introducing recombinant bacteria. Bait stations could be used to introduce the recombinant bacteria into the wild population, but each generation of adults would have to be inoculated, increasing the costs of using this method to control malaria. Even with the additional costs of inoculating each generation, this method of controlling malaria may be a preferred technique over using traditional methods of insecticides and drugs by reducing the number of individuals infected with malaria and also by reducing the environmental damage that can occur from insecticides.

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